 DOI 10.1099/mic.0.28077-0

The sigma factor RpoS is required for stress tolerance and environmental fitness of *Pseudomonas fluorescens* Pf-5

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Received 29 March 2005
Revised 16 June 2005
Accepted 25 June 2005

Many micro-organisms exist in natural habitats that are subject to severe or dramatically fluctuating environmental conditions. Such is the case for bacteria inhabiting plant surfaces, where they are exposed to UV irradiation, oxygen radicals, and large fluctuations in temperature and moisture. This study focuses on the role of RpoS, a central regulator of stationary-phase gene expression in bacterial cells, in stress response and environmental fitness of *Pseudomonas fluorescens* Pf-5. Strain Pf-5 is a rhizosphere-inhabiting bacterium that suppresses plant diseases caused by several plant-pathogenic fungi and oomycetes. Previous studies demonstrated that *rpoS* was required for osmotic and oxidative stress resistance of Pf-5. The results of this study demonstrate a role for *rpoS* in tolerance of Pf-5 to freezing, starvation, UV irradiation and desiccation stress. In field studies, an *rpoS* mutant was compromised in rhizosphere colonization of plants in dry soil, whereas similar rhizosphere populations were established by Pf-5 and an *rpoS* mutant in well-irrigated soils. RpoS is a key determinant in stress response and environmental fitness of the rhizosphere bacterium *P. fluorescens* Pf-5.

INTRODUCTION

Fluorescent pseudomonads are common inhabitants of plant surfaces, where they are exposed to severe or fluctuating environmental conditions. Little is known about the mechanisms by which these bacteria adapt to stresses encountered in the environment, but it is generally accepted that bacterial cells exist, at least for a portion of their lives on plant surfaces, in a nutrient-limited state. Gram-negative bacteria, in response to starvation or upon entry into stationary phase, undergo complex morphological and physiological changes that result in cells that are metabolically less active and more resistant to environmental and physiological stresses, than exponential-phase cells (Givskov *et al.*, 1994; van Veen *et al.*, 1997; Nyström, 2004).

In *Escherichia coli*, the sigma factor σ^S (or σ^{38}), encoded by *rpoS*, is a central regulator of gene expression by cells in stationary phase (Hengge-Aronis, 2002a, b; Kolter *et al.*, 1993). Under conditions of cellular starvation, σ^S accumulates, binds and directs the RNA polymerase holoenzyme to over 100 genes constituting the RpoS regulon (Ishihama, 2000). Members of the regulon encode proteins with diverse functions, including stress response. Consequently, a mutation in *rpoS* has pleiotropic effects on cells in stationary phase, including diminished survival when subjected to environmental stresses (Hengge-Aronis, 2002a, b; Kolter *et al.*, 1993). Many of the temporal changes in cell morphology and physiology that occur during the transition from exponential growth to stationary phase are similar in *E. coli* and *Pseudomonas* spp. For example, members of both genera exhibit reductions in cell size, DNA and ribosome content, and the rate of protein synthesis upon entry to stationary phase (Venturi, 2003). In *Pseudomonas* spp., as in *E. coli*, σ^S is required for optimal survival of stationary-phase cells exposed to various environmental stresses (Heeb *et al.*, 2005; Jørgensen *et al.*, 1999; Miller *et al.*, 2001a, b; Miura *et al.*, 1998; Ramos-González & Molin, 1998; Sarniguet *et al.*, 1995; Suh *et al.*, 1999; Whistler *et al.*, 2000). Nevertheless, recent studies imply that fundamental differences in the molecular events characterizing the transition between exponential growth and stationary phase may exist between the two genera (Venturi, 2003).

The rhizosphere inhabitant *Pseudomonas fluorescens* strain Pf-5 is a biological control organism that suppresses plant diseases caused by soil-borne fungi and oomycetes (Howell & Stipanovic, 1979). Pf-5 produces an array of secondary metabolites, including the antibiotics pyoluteorin (Howell & Stipanovic, 1980), 2,4-diacetylphloroglucinol (Nowak-Thompson *et al.*, 1994) and pyrrolnitrin (Howell &

Abbreviations: AUPC, area under the population curve; RAUPC, relative area under the population curve.

Stipanovic, 1979), that are toxic to soilborne plant pathogens. Previous studies have demonstrated that an *rpoS* mutant of Pf-5 is altered in secondary metabolite production, overproducing pyoluteorin and 2,4-diacetylphloroglucinol, and lacking production of pyrrolnitrin (Pfender *et al.*, 1993; Sarniguet *et al.*, 1995). In addition to producing antibiotics, effective biological control organisms must colonize plant surfaces, where they encounter a myriad of environmental stresses. RpoS clearly has an important role in stress response of the biological control agent Pf-5, as an *rpoS* mutant is more sensitive than the parental strain to osmotic and oxidative stresses (Sarniguet *et al.*, 1995). Therefore, it is not surprising that *rpoS* is required for optimal survival of Pf-5 on surfaces of seeds (Sarniguet *et al.*, 1995) and wheat straw maintained under controlled experimental conditions (Pfender *et al.*, 1993).

The current study was initiated to expand our understanding of the role of *rpoS* in the stress response of *P. fluorescens* Pf-5. We demonstrated that *rpoS* has a role in the survival of Pf-5 exposed to starvation, freezing, desiccation and UV irradiation, all of which are likely to be encountered by bacteria inhabiting plant surfaces. The role of *rpoS* in establishment of Pf-5 on seed surfaces was demonstrated previously in experiments done in an environmentally controlled growth chamber (Sarniguet *et al.*, 1995), but results from such experiments are not always predictive of those done in a field environment. Bacteria inhabiting plant surfaces in the field are influenced by many factors that are not reproduced in growth chambers, including differences in the physiology and growth of plants, environmental constraints imposed by variable weather patterns, and the presence of indigenous organisms. Therefore, in this study, we demonstrate that *rpoS* also has a role in the establishment and survival of Pf-5 in the rhizosphere under field conditions.

METHODS

Bacterial strains. *P. fluorescens* strain Pf-5 (JL4474) was isolated from the cotton rhizosphere by C. Howell, United States Department of Agriculture (Howell & Stipanovic, 1979). An *rpoS* mutant of Pf-5 (Pf-5 *rpoS*, JL3985), selected after Tn*5* mutagenesis, was described previously (Pfender *et al.*, 1993; Sarniguet *et al.*, 1995). A complemented strain (Pf-5 *rpoS*$^+$, JL4391) was generated previously by introducing pJEL5649, a stable multicopy plasmid containing a 2·9 kb genomic fragment of Pf-5 encompassing *rpoS*, into JL3985 (Sarniguet *et al.*, 1995).

In field experiments, a spontaneous mutant of Pf-5 resistant to rifampicin (Pf-5R, JL3871) was used to facilitate estimates of rhizosphere population size. Pf-5R is similar to the parental strain in growth rate and production of secondary metabolites (data not shown). An *rpoS* mutant of Pf-5R (Pf-5R *rpoS*, JL4739) was generated for this study by marker-exchange mutagenesis. Briefly, an *Eco*RI genomic fragment containing *rpoS*::Tn*5* of JL3985 was cloned into pBR322 to generate pJEL1995 (Pfender *et al.*, 1993). Marker-exchange mutagenesis of Pf-5R with DNA of pJEL1995 was performed as described previously (Whistler *et al.*, 2000). Southern analysis was used to determine that the resultant rifampicin-resistant, kanamycin-resistant strain (Pf-5R *rpoS*, JL4719) contains a single genomic Tn*5* insertion, which is in *rpoS*. Like the *rpoS* mutant of Pf-5 (Sarniguet *et al.*, 1995), Pf-5R *rpoS* was found to be prototrophic, lacking detectable tryptophan-sidechain oxidase activity, lacking pyrrolnitrin production, and overproducing pyoluteorin and 2,4-diacetylphloroglucinol (data not shown). The response of Pf-5R *rpoS* to oxidative and freezing stress was evaluated prior to the field experiments, and its stress response was found to be similar to that of the *rpoS* mutant of Pf-5 (data not shown). Bacterial strains were maintained in nutrient broth containing 15 % (v/v) glycerol at −80 °C.

Culture conditions. Pf-5 and derivatives were grown in Luria–Bertani broth (LB) at 27 °C on a rotary shaker (200 r.p.m.). Exponential-phase cells were harvested from cultures after 6 h incubation, whereas stationary-phase cells were collected from cultures 4 h after the OD_{600} of cultures stopped increasing (i.e. they entered stationary phase), as described previously (Sarniguet *et al.*, 1995). Bacterial cells were collected by centrifugation (5000 **g**, 2 min), washed in 10 mM phosphate buffer (pH 7·0), and suspended to a final density of $\sim 1 \times 10^8$ c.f.u. ml^{-1} in buffer, unless stated otherwise.

For field experiments, bacterial strains were cultured for 3 days at 20 °C on Pseudomonas agar F (PAF; Difco) containing 50 μg rifampicin ml^{-1}, or 50 μg rifampicin ml^{-1} and 50 μg kanamycin ml^{-1}, for culture of Pf-5R or Pf-5R *rpoS*, respectively. Cells were scraped from the surface of the agar, and suspended in phosphate buffer. Each suspension was adjusted to $\sim 1 \times 10^{10}$ c.f.u. ml^{-1} prior to treatment of plant materials.

Stress tolerance assays. Bacterial cells were cultured in broth as described above. After exposure to stresses imposed in the laboratory, dilutions of cells were spread on PAF, or PAF with 50 μg kanamycin ml^{-1}, for enumeration of Pf-5 or Pf-5 *rpoS*, respectively. Each assay contained at least three replicates of each strain, and all experiments were repeated at least three times.

Tolerance to UV irradiation was measured by methods described by Whistler *et al.* (2000). Briefly, dilutions of cell suspensions were spread onto PAF, and immediately exposed to UV irradiation (λ 254 nm) at doses up to 160 J m^{-2}. After UV irradiation, plates were incubated in darkness for 3 days; afterwards, colonies were counted.

Survival of starvation stress was assessed by incubating stationary-phase cells in 1 mM sterile potassium phosphate buffer (pH 7) containing 0·8 % (w/v) NaCl, with agitation (200 r.p.m.) at 27 °C for up to 36 days.

Two methods were used to determine the sensitivity of Pf-5 and derivatives to desiccation stress. In the first method (Stockwell *et al.*, 1998), 10 μl samples of bacterial cells suspended in sterile distilled water (10^8 c.f.u. ml^{-1}) were placed on 1 cm^2 sterilized Whatman no. 1 filter paper. The filters were suspended over a saturated solution of NaCl in sealed containers held at 25 °C to maintain a relative humidity of 75 % (Lindow *et al.*, 1993). Control sets of filters were suspended in chambers containing water and 100 % relative humidity. Periodically, filters were removed and placed in tubes containing 10 mM phosphate buffer (pH 7·0), and tubes were placed in an ultrasonic bath for 3 min prior to spreading dilutions on PAF. In a second desiccation stress assay, 10 μl samples of bacterial suspensions in water or 100 mM trehalose were spotted on autoclaved 1 cm^2 glass squares, and placed in a sterile transfer hood, with the blower generating an air flow of 2 km h^{-1}. Glass squares were removed periodically, and culturable bacterial populations were enumerated as described for the desiccation assay with filter paper.

Tolerance of mild heat shock was measured by exposure of 100 μl bacterial suspensions to 42 °C for 5 or 20 min. After heating, 900 μl phosphate buffer (20 °C) was added, and the suspension was mixed using a vortex for 30 s prior to dilution plating.

To determine the relative tolerance of Pf-5 and derivatives to freezing, 100 μl samples of bacterial suspensions in water or 100 mM trehalose (Sigma) were incubated at −20 or −80 °C. After 12–18 h, 900 μl phosphate buffer (20 °C) was added to rapidly thaw the frozen suspension. Samples were mixed using a vortex for 30 s, prior to dilution plating.

Colonization of bean seeds and emerging roots in growth chambers. Bean (*Phaseolus vulgaris* cv. 'Bush Blue Lake 274') seeds were surface disinfected by soaking in 70 % ethanol for 1 min, and a 1 % hypochlorite solution for 15 min, followed by three rinses in sterile distilled water. Seeds were placed on sterile damp filter paper for 36 h at room temperature. Pre-germinated seeds were soaked in 2×10^{8} c.f.u. ml^{-1} suspensions of Pf-5 or Pf-5 *rpoS* for 10 min, prior to planting in individual pots containing 20 g pasteurized soil (Newberg fine sandy loam that passed through a 2 mm screen). Water was added to approximate a soil matric potential of −0·02 MPa (moist soil) or −1·0 MPa (dry soil), as determined from a soil moisture release curve. Pots were arranged in a randomized complete block, and maintained in a growth chamber at 20 °C without light. Ten seeds or seedlings for each soil moisture and bacterial treatment were harvested 1, 2, 3 and 5 days after planting. Plant material and adhering soil were placed in 5 ml 10 mM phosphate buffer, and subjected to vibrations in an ultrasonic cleaner for 5 min. Culturable populations of bacteria from the surface of bean seedlings were estimated by spreading dilutions of seed or seedling washes on PAF for recovery of Pf-5, or PAF containing 50 μg kanamycin ml^{-1} for enumeration of Pf-5 *rpoS*.

Survival in the rhizosphere in field plots. Field plots were established at the Oregon State University Botany and Plant Pathology Experimental Farm in Corvallis on 6 July 1997, 11 September 1997, and 26 July 1998. The soils in this area are characterized as Newberg fine sandy loam. Plots were split according to water application. 'Dry' soil blocks were hoed daily for 3 days prior to planting. 'Moist' soil blocks also were hoed, but water was added to saturation daily for 3 days prior to planting. Soil moisture content was estimated at each sample time by gravimetric methods. The soil moisture content of 'dry' soil at the time of planting was below −1·5 MPa, which corresponded to 3·6 % (w/w) in July 1997, 5·7 % in September 1997, and 4·3 % in 1998. The soil moisture content for the 'moist' blocks at the time of planting was ∼−0·04 to −0·02 MPa (15·9 %, w/w, in July 1997; 18 % in September 1997; and 21 % in 1998). Weather data were obtained from an AgriMet weather station (Northwest Cooperative Agricultural Weather Network, Bonneville Power Administration, and the US Bureau of Reclamation, Boise, Idaho) located near the plot. The mean daily air temperature was 18 °C in July 1997, 15 °C in September 1997, and 21 °C during the 1998 experiment. In July 1997, rain was measured on days 3–5; the total rainfall was ∼5 mm. Rain was frequent during the experiment in September 1997. Measurable rain fell daily from 3 days after planting, to 8 days after planting; the total rainfall during that period was ∼75 mm. July 1998 was dry, with only one light rain event (1 mm) during the experimental period.

Bean seeds (cv. 'Bush Blue Lake 274') were surface disinfected, and incubated at room temperature on sterile moist paper towels overnight. The three bacterial treatments were: (1) Pf-5R (1×10^{10} c.f.u. ml^{-1}), (2) Pf-5R *rpoS* (1×10^{10} c.f.u. ml^{-1}), and (3) a 1 : 1 mixture of Pf-5R and Pf-5R *rpoS* (each strain at 5×10^{9} c.f.u. ml^{-1}). Bacteria were applied by soaking bean seeds for 10 min in cell suspensions. Immediately after treatment, bean seeds were planted in moist and dry soil plots that were arranged in a randomized complete block design with five (1997) or six (1998) replications per bacterial strain per soil treatment. Each replicate plot contained 100 treated bean seeds in a 2 m × 0·25 m area, surrounded by a 1 m zone of fallow soil. After planting, moist-soil plots were watered every 2 days. In July 1997, dry-soil plots were watered 6 days after planting. In 1998, dry-soil plots were watered 7 days after planting.

Periodically, five seedlings per plot were harvested by placing the seeds and individual root systems with adhering soil in 5 or 10 ml 10 mM phosphate buffer, and subjecting samples to vibrations in an ultrasonic cleaner for 5 min. Culturable populations of bacteria from the surface of bean seedlings were estimated by spreading dilutions on PAF containing 100 μg rifampicin ml^{-1}, or PAF 100 μg containing rifampicin ml^{-1} and 50 μg kanamycin ml^{-1}, for recovery of Pf-5R or Pf-5R *rpoS*, respectively. Cycloheximide (50 μg ml^{-1}) was added to each medium to inhibit fungal growth.

Data analysis. Mean population size of bacterial strains and standard error were calculated by averaging the $\log_{10}$ c.f.u. obtained from each sample. For samples with a non-detectable bacterial population size, a value of the detection limit minus 1 c.f.u. was assigned to the sample. The relative area under the population curve (RAUPC) was calculated for bacterial populations recovered from plant surfaces to compare differences in growth of bacterial strains (Stockwell *et al.*, 2002). The area under the population curve (AUPC) was calculated for each trial with the following formula:

$$\mathrm{AUPC}=\sum_{i=1}^{n}[(y_i+y_{i-1})/2]\cdot(t_i-t_{i-1})$$

where y is the mean population size of a bacterial strain on the ith day after planting, and t is the corresponding sample time. Because the length of the sample period varied among trials, the AUPC was converted to the RAUPC by dividing the AUPC by the number of days during the sampling period (Stockwell *et al.*, 2002).

For analysis of data from laboratory experiments assessing stress tolerance, survival ratios were calculated by dividing the observed population at a sample point by the initial population. Survival ratios were $\log_{10}$ transformed prior to statistical analyses. All population data, including RAUPC, were subjected to analysis of variance and mean separation by Fisher's protected least significant difference test at $P=0\cdot05$ using Statistical Analysis Systems (SAS Institute).

RESULTS

RpoS influences the sensitivity of stationary-phase cells of Pf-5 to diverse environmental stresses

Stationary-phase cells of the *rpoS* mutant were less tolerant of UV radiation than were stationary-phase cells of Pf-5, or the complemented mutant Pf-5 *rpoS*$^{+}$ (Fig. 1a). Low doses (∼40 J m^{-2}) of UV radiation significantly reduced the numbers of culturable cells of the *rpoS* mutant, and exponential-phase cells of Pf-5. In contrast, a UV irradiation dose of ∼60 J m^{-2} was required to significantly reduce culturability of stationary-phase cells of Pf-5, and the complemented mutant Pf-5 *rpoS*$^{+}$.

Tolerance of starvation stress was assessed over 1 month by suspending stationary-phase cells of Pf-5 or the *rpoS* mutant in sterile, aerated PBS. In each of four experiments, Pf-5 maintained significantly greater culturable populations than did the *rpoS* mutant (Fig. 1b). Over the course of the experiment, the culturable population size of Pf-5

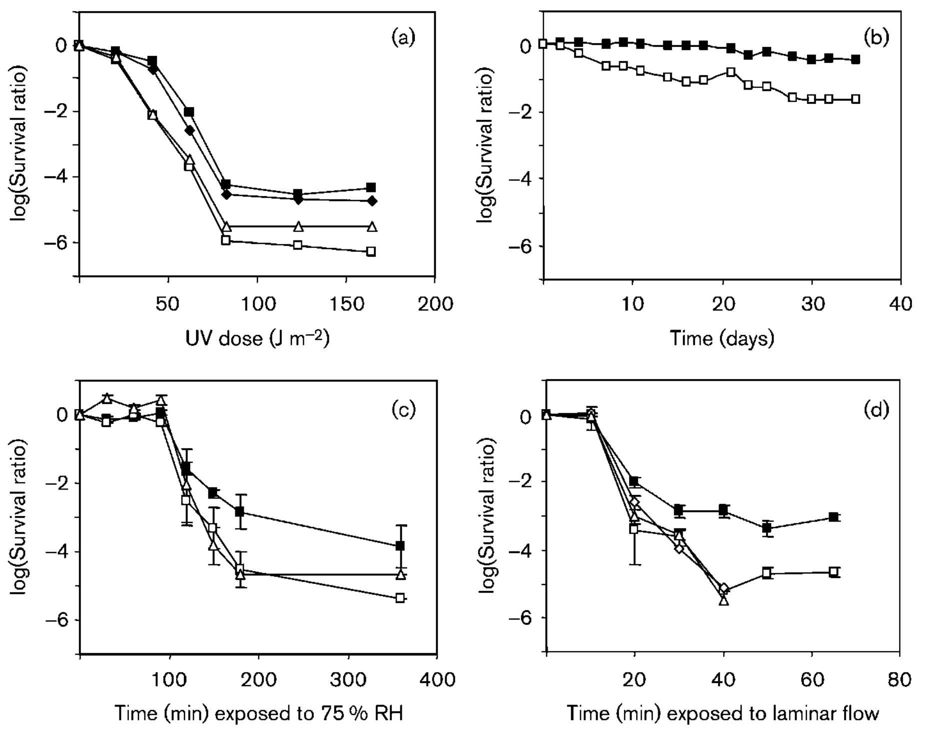

Fig. 1. Effect of *rpoS* on stress tolerance of *P. fluorescens* Pf-5. Stress tolerance of stationary-phase cells of Pf-5 (■), its *rpoS* mutant Pf-5 *rpoS* (□), and a complemented mutant Pf-5 *rpoS*$^+$ (◆), was assessed. Additional tests were conducted on exponential-phase cells of Pf-5 (△) and Pf-5 *rpoS* (◇). Cells were exposed to UV irradiation (a), starvation by suspension in saline (b), desiccation on paper at 75 % relative humidity (RH) (c), and desiccation during drying in laminar airflow hood (d). The proportion of cells culturable after exposure was estimated by spreading dilutions of samples onto PAF. Culturability is expressed as the logarithm (base 10) of the survival ratio, which is the population size after a stress exposure period or dose divided by the population size in the initial sample. Vertical bars represent one standard error of the mean; they are sometimes obscured by the symbols. The results shown are from single experiments; each experiment was repeated at least twice, and yielded similar responses to those shown in the figure.

decreased by 0·5 log units, whereas culturable populations of the *rpoS* mutant decreased by 1–1·5 log units.

Desiccation stress was imposed in two ways: by placing cell suspensions on filter paper in sealed chambers with controlled humidity, and on glass surfaces dried more rapidly in a transfer hood. Pf-5 and the *rpoS* mutant maintained high populations for 6 h on filter papers maintained in sealed chambers at 100 % relative humidity (data not shown). At 75 % relative humidity, however, culturable populations decreased as the filters dried (Fig. 1c). Exponential-phase cells of Pf-5, and stationary-phase cells of the *rpoS* mutant, were significantly more sensitive to desiccation at 75 % relative humidity than were stationary phase cells of Pf-5 (Fig. 1c). When dried more rapidly on glass surfaces, populations of stationary-phase cells of Pf-5 or the complemented mutant Pf-5 *rpoS*$^+$ decreased by 3 log units during the 65 min drying period, whereas populations of exponential-phase cells of Pf-5, and cells of the *rpoS* mutant, decreased by ~5 log units (Fig. 1d). If cells of Pf-5 and the *rpoS* mutant were suspended in trehalose before drying on glass squares, they maintained stable culturable population sizes (data not shown). After 2 h exposure in a transfer hood, culturable populations of Pf-5 and Pf-5 *rpoS* suspended in trehalose decreased a mean log survival ratio of −1·1; whereas culturable cells of either strain suspended in water before drying were not detected (log survival ratio $<-6{\cdot}5$, data not shown).

Cells of Pf-5 and Pf-5 *rpoS* were tolerant of mild heat shock. After exposure to 42 °C for 5 or 20 min, culturable population sizes of Pf-5 and Pf-5 *rpoS* did not differ significantly from each other, or from the initial population size before treatment (data not shown).

When stationary-phase cells of Pf-5 were exposed to temperatures of −20 or −80 °C, culturable populations decreased from 2×10^8 to 1×10^7 c.f.u. ml^{-1} (Fig. 2). Stationary-phase cells of the *rpoS* mutant were more sensitive than Pf-5 to low temperatures (Fig. 2); among experiments, culturable populations of Pf-5 *rpoS* ranged from $2{\cdot}5\times10^4$ to $6{\cdot}3\times10^6$ c.f.u. ml^{-1} after treatment at −80 °C, and from $3{\cdot}2\times10^2$ to $1{\cdot}3\times10^5$ c.f.u. ml^{-1} at −20 °C. Exponential-phase cells of Pf-5 were more

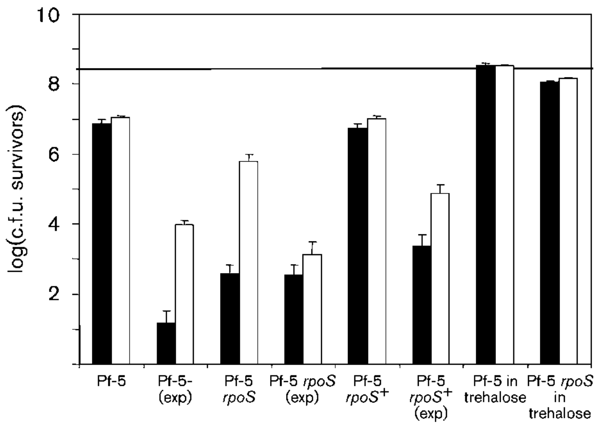

Fig. 2. Effect of *rpoS* on tolerance to freezing of *P. fluorescens* Pf-5. Freezing tolerance of stationary-phase cells and exponentially growing cells (exp) of Pf-5, its *rpoS* mutant Pf-5 *rpoS*, and a complemented mutant Pf-5 $rpoS^{+}$, was assessed. Bacterial suspensions (100 μl; 2×10^{8} c.f.u. ml^{-1}) in water or 100 mM trehalose were exposed to −20 °C (black bars) or −80 °C (white bars) for 18 h. The bars represent the mean of the logarithm-transformed (base 10) population size of culturable survivors of freezing and thawing. Vertical lines represent one standard error of the mean. The horizontal line represents the initial population size before freezing. The results shown are from a single representative experiment; this experiment was repeated three times, and yielded similar responses.

sensitive than stationary-phase cells to exposure at −20 or −80 °C; mean population sizes of survivors were $\leqslant 2\times10^{4}$ c.f.u. ml^{-1}. Similar to exponential-phase cells of Pf-5, exponential-phase cells of the *rpoS* mutant also were sensitive to freezing; mean populations of survivors ranged between 2×10^{2} and 2×10^{3} c.f.u. ml^{-1} (Fig. 2). In all experiments, survival of the complemented mutant Pf-5 $rpoS^{+}$ did not differ significantly from survival of the wild-type strain. For stationary-phase or exponential-phase cells of all strains, treatment with trehalose prior to freezing resulted in no significant loss in culturability (Fig. 2), presumably because of the cryoprotectant properties of trehalose (Zavaglia *et al.*, 2003).

Reduced fitness of the *rpoS* mutant in the bean spermosphere

Both Pf-5 and the *rpoS* mutant colonized the spermosphere of bean when planted in moist pasteurized soil. Over time, mean bacterial populations increased by 1–4 log units on bean seeds in moist soil (Fig. 3a, c). Populations of Pf-5 also increased by 1–2 log units on bean seeds planted in dry pasteurized soil (Fig. 3b, d). In contrast, the population of Pf-5 *rpoS* increased for 1–2 days after planting, then decreased (Fig. 3b, d). The parental strain Pf-5 did not exhibit a precipitous population decline on bean seeds in dry soils, and its mean population sizes were significantly higher than those of Pf-5 *rpoS* by the end of the experiments

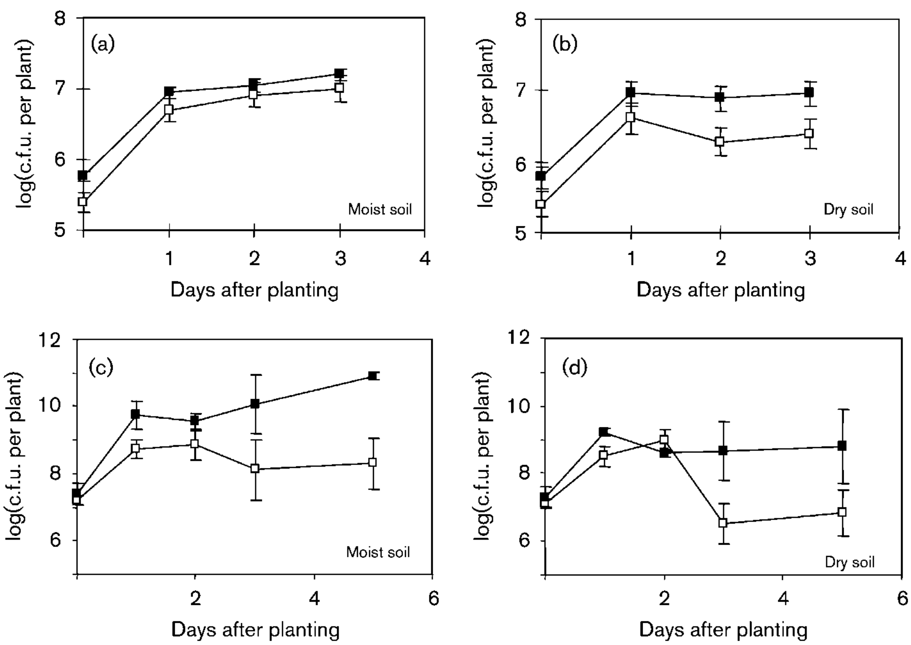

Fig. 3. Effect of *rpoS* on survival of Pf-5 in the rhizosphere of beans planted in moist and dry pasteurized soils. Surface-sterilized pre-germinated bean seeds were soaked in 2×10^{8} c.f.u. ml^{-1} Pf-5 or Pf-5 *rpoS*, and planted in pasteurized Newberg fine sandy loam at a soil matric potential of −0·02 MPa (moist soil; a, c) or −1·0 MPa (dry soil; b, d) and incubated at 20 °C. Culturable populations of Pf-5 (■) and Pf-5 *rpoS* (□) from bean seedlings were estimated from ten replicate plants. (a, b) Data from one experiment. (c, d) Data from a second experiment. Vertical bars represent one standard error of the mean.

Table 1. Mean RAUPC for Pf-5 and the *rpoS* mutant on bean seeds planted in dry or moistened pasteurized soils, and maintained in growth chambers

Growth chamber experiments were arranged in a randomized complete block design with ten replications per treatment. Bean seeds were suspended in 2×10^8 c.f.u. ml^{-1} of Pf-5 or its *rpoS* mutant for 10 min before planting into moist soil (−0·02 MPa) or dry soil (1·0 MPa) maintained at 20 °C. The RAUPC was calculated for bacterial populations on seeds and seedlings sampled over 5 days.

Soil treatment and strain	Mean RAUPC*	
	Trial 1	Trial 2
Moist soil		
Pf-5	10·2[a]	10·2[a]
Pf-5 *rpoS*	8·8[b]	8·4[b]
Dry soil		
Pf-5	9·7[a]	8·8[b]
Pf-5 *rpoS*	8·1[c]	7·6[c]

*Means within a column followed by the same letter are not significantly different by Fisher's protected least significance difference at $P=0{\cdot}05$.

(Fig. 3b, d). At both soil moistures, the RAUPC of Pf-5 was consistently greater than the RAUPC of Pf-5 *rpoS* (Table 1). The RAUPC of the *rpoS* mutant was significantly greater on bean seeds planted in moist soil compared with dry soil (Table 1).

Reduced fitness of the *rpoS* mutant in the spermosphere and rhizosphere of bean in the field

Soil moisture content influenced the population dynamics of Pf-5R and Pf-5R *rpoS* in the spermosphere and rhizosphere of bean in each of three field trials, with both strains consistently establishing larger populations on beans planted in moist soils compared with those in dry soils (Fig. 4). In two trials, Pf-5R established and maintained larger populations on bean planted in moist soils than did Pf-5R *rpoS* (Fig. 4a, b; Table 2).

Populations of Pf-5R and its *rpoS* mutant decreased significantly within 1 day after planting in dry soil (Fig. 4). Populations of both strains remained low in dry soil plots until the first rain or watering event. Populations of Pf-5R and its *rpoS* mutant then increased over time until they were similar to those obtained from plants in moist soil plots (Fig. 4). The mean culturable populations of Pf-5R were greater than those of Pf-5R *rpoS* during the population decline phase after planting (Fig. 4). In dry soils, Pf-5R exhibited a greater RAUPC than its *rpoS* mutant in every experiment (Table 2). The differences in populations of Pf-5R and its *rpoS* mutant on bean were evident mainly after planting in dry soil.

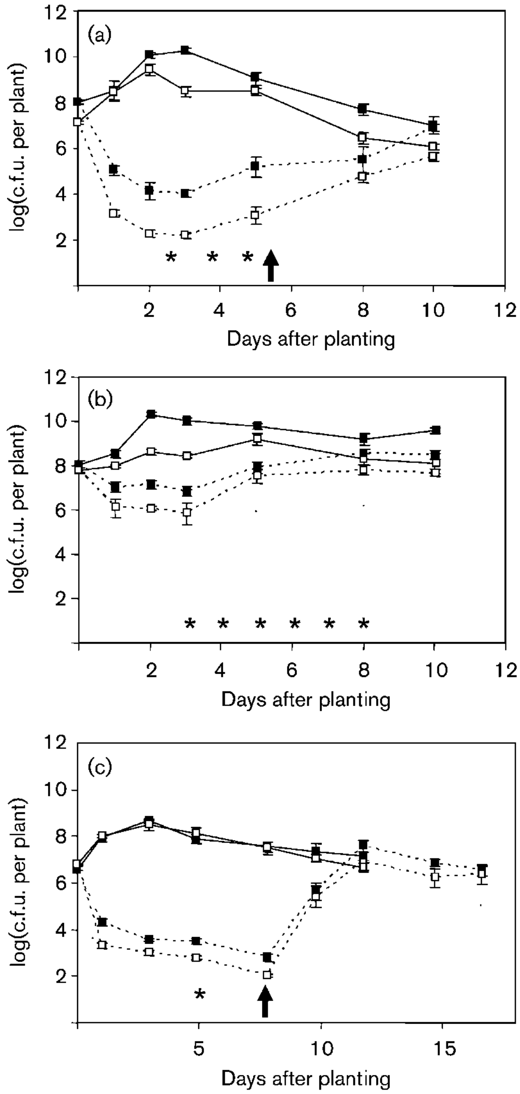

Fig. 4. Effect of *rpoS* on survival of Pf-5 in the rhizosphere of beans planted in moist and dry field soils. Surface-sterilized bean seeds were soaked in 1×10^{10} c.f.u. ml^{-1} of Pf-5R or Pf-5R *rpoS*, and planted in soil blocks that differed in soil matric potential (moist soil ∼−0·03 MPa, and dry soil ∼−1·5 MPa) in July 1997 (a), September 1997 (b), and July 1998 (c). Plots were arranged in a randomized complete block design with five (a, b) or six (c) replications per bacterial strain per soil treatment. Culturable populations of Pf-5R (■) or Pf-5R *rpoS* (□) from bean seedlings were estimated by spreading dilutions of bean washes on selective media. Symbols connected with continuous lines represent populations recovered from bean planted in moist soil, and those connected with dashed lines are populations from bean planted in dry soil. Vertical bars represent one standard error of the mean. Asterisks above the *x*-axis represent days with measurable rain. The arrow on the *x*-axis represents the day that dry plots (a, c) were watered to field capacity.

In experiments in 1997, Pf-5R and its *rpoS* mutant also were co-inoculated onto the same bean seeds. The patterns of

Table 2. Mean RAUPC for Pf-5R and Pf-5R *rpoS* on bean planted in field plots containing dry soils, or soils at field capacity

Plots were arranged in a complete randomized block design, with five or six replications per treatment. Bean seeds were suspended in 1×10^{10} c.f.u. ml^{-1} Pf-5R or its *rpoS* mutant for 10 min before planting into moist soil ($\sim-0{\cdot}03$ MPa) or dry soil ($\sim-1{\cdot}5$ MPa). Periodically, plants were harvested, and bacterial populations were enumerated by dilution plating. The RAUPC was calculated for bacterial populations on seed and seedling samples taken from day 0 to day 12.

Soil treatment and strain	Mean RAUPC*		
	Jul 1997	Sep 1997	Jul 1998
Moist soil			
Pf-5R	$8{\cdot}7^{a}$	$9{\cdot}4^{a}$	$7{\cdot}8^{a}$
Pf-5R *rpoS*	$7{\cdot}8^{b}$	$8{\cdot}4^{b}$	$7{\cdot}8^{a}$
Dry soil			
Pf-5R	$5{\cdot}3^{c}$	$7{\cdot}7^{c}$	$4{\cdot}4^{b}$
Pf-5R *rpoS*	$3{\cdot}7^{d}$	$7{\cdot}0^{d}$	$3{\cdot}7^{c}$

*Means within a column followed by the same letter are not significantly different by Fischer's protected least significance difference at $P=0{\cdot}05$.

Table 3. Mean RAUPC for Pf-5R and Pf-5R *rpoS* co-inoculated on bean seeds, and planted in field plots containing dry soils or soils at field capacity

Plots were arranged in a randomized complete block design with five replications per treatment. Bean seeds were suspended in 5×10^{9} c.f.u. ml^{-1} of Pf-5R and its *rpoS* mutant for 10 min before planting into moist soil ($\sim-0{\cdot}03$ MPa) or dry soil ($\sim-1{\cdot}5$ MPa). The RAPUC was calculated for bacterial populations recovered from all seed and seedling sample dates.

Soil treatment and strain	Mean RAUPC*	
	Jul 1997	Sep 1997
Moist soil		
Pf-5R	$8{\cdot}1^{a}$	$8{\cdot}8^{a}$
Pf-5R *rpoS*	$7{\cdot}3^{b}$	$8{\cdot}1^{b}$
Dry soil		
Pf-5R	$4{\cdot}8^{c}$	$6{\cdot}9^{c}$
Pf-5R *rpoS*	$3{\cdot}6^{d}$	$6{\cdot}6^{c}$

*Means within a column followed by the same letter are not significantly different by Fisher's protected least significance difference at $P=0{\cdot}05$.

population increase or decrease of co-inoculated strains were similar to those shown in Fig. 4 for single-strain inoculants (data not shown). The RAUPC of Pf-5R on beans in moist soil was significantly greater in each trial compared with Pf-5R or Pf-5R *rpoS* on seed in dry soil (Table 3). Similar to results with single-strain inoculants, the RAUPC of Pf-5R *rpoS* on seed co-treated with Pf-5R was significantly lower than its co-inoculant in repeated experiments in moist soil conditions, and also on seed in dry soil in one of the two trials (Table 3).

DISCUSSION

This study established the sigma factor RpoS as a key determinant in the fitness of *P. fluorescens* in the rhizosphere of plants in the field. Furthermore, *rpoS* was shown to have a key role in survival of bacterial cells exposed to starvation, freezing, desiccation and UV irradiation, in addition to its established role in oxidative and osmotic stress response, which was demonstrated earlier (Sarniguet *et al.*, 1995). Because plant-associated bacteria are exposed to harsh and fluctuating environmental conditions on field-grown plants, the capacity to withstand exposure to such stresses is likely to contribute to their fitness in these natural habitats.

The capacity to survive periods of desiccation is an important fitness factor in plant-associated bacteria, and the results of this study indicate that *rpoS* plays a key role in that capacity. This finding is consistent with previous observations that stationary-phase cells of Gram-negative bacteria are more desiccation resistant than are exponential-phase cells (Potts, 1994). In *Pseudomonas putida*, 14 of 35 genes identified on the basis of their induction under matric stress are preferentially expressed by stationary-phase cells rather than exponential-phase cells (Van de Mortel & Halverson, 2004). Many of these 'water-deprivation-controlled' genes are required for maximum survival of stationary-phase cells exposed to desiccation stress, an observation that prompted Van de Mortel & Halverson (2004) to speculate that the genes may be under the control of *rpoS*. In response to desiccation, microbial cells undergo physiological changes that are likely to optimize their survival under these conditions. The physiological changes include the accumulation of disaccharides, such as trehalose, which serve as protectants of macromolecules within the cell; the accumulation of water-stress proteins; alterations in lipid composition of membranes to maintain membrane fluidity; enhanced production of extracellular polysaccharides; and morphological changes that maximize volume-to-surface ratios of bacterial colonies (Potts, 1994). The mechanisms by which RpoS influences desiccation stress response of *P. fluorescens* are unknown, and could include the control of one or more of these physiological processes. Sigma factors other than RpoS also are known to play a role in the adaptation of *P. fluorescens* to desiccation stress. The sigma factor AlgU (also called AlgT, RpoE and σ^{22}), a key determinant of extracellular polysaccharide production, is required for optimal survival of *P. fluorescens* CHA0 when exposed to desiccation and osmotic stress (Schnider-Keel *et al.*, 2001). An *rpoN* mutant of CHA0 is also more sensitive than the parental strain to salt stress, indicating a role for σ^{54} in osmotic stress (Péchy-Tarr *et al.*, 2005). It is likely that the desiccation stress response is a complex physiological process, components of which are subject to control by different regulatory circuits in *Pseudomonas* spp.

The results of this study also establish a significant role for *rpoS* in the capacity of Pf-5 to survive freezing and thawing. RpoS had a positive influence on the ability of Pf-5 to survive freezing at both −80 and −20 °C, temperatures that differentially influenced the survival of the cells. Trehalose served as a protectant for cells of Pf-5 exposed to freezing and to desiccation, a phenomenon that has been attributed to a dual mechanism of trehalose in other micro-organisms: stabilization of membranes and proteins by replacing water, and preservation of the intracellular water structure (Tanghe *et al.*, 2003).

In addition to the role of *rpoS* in desiccation and freezing survival, which has not been reported in other Gram-negative bacteria, *rpoS* was required for optimal survival of UV irradiation by *P. fluorescens* Pf-5. This finding is consistent with the significant role of *rpoS* in survival of the plant pathogen *Pseudomonas syringae* when exposed to UV irradiation (Miller *et al.*, 2001b). *rpoS* also was required for optimal survival of Pf-5 when exposed to starvation stress, and a similar role for *rpoS* was reported for *P. putida* KT2440 (Ramos-González & Molin, 1998). In *Pseudomonas aeruginosa*, the role of *rpoS* in starvation stress response appears to be more subtle; it was found to influence the rate of die-off but not the final level of survival in one study (Jørgensen *et al.*, 1999), and to be affected by media in a second study (Suh *et al.*, 1999). An apparent discrepancy in the role of *rpoS* in stress response of different *Pseudomonas* spp. could be inferred by comparing studies evaluating survival of heat stress, with *rpoS* playing a significant role in survival of heat shock in *P. aeruginosa* (Jørgensen *et al.*, 1999; Suh *et al.*, 1999) that was not observed in the current study evaluating *P. fluorescens* Pf-5. This difference may be explained by different experimental conditions: *P. aeruginosa* was exposed to 50 or 53 °C in previous studies, whereas *P. fluorescens* was exposed to 42 °C in the current study, a temperature selected to approximate the maximum that Pf-5 might experience in a soil environment. *rpoS* is known to play a critical role in the oxidative stress response of *P. aeruginosa* (Jørgensen *et al.*, 1999; Suh *et al.*, 1999), *P. fluorescens* (Heeb *et al.*, 2005; Sarniguet *et al.*, 1995) and *P. putida* (Miller *et al.*, 2001a; Miura *et al.*, 1998), and the osmotic stress response of *P. aeruginosa* (Jørgensen *et al.*, 1999; Suh *et al.*, 1999) and *P. fluorescens* (Sarniguet *et al.*, 1995). While strain differences are likely to be revealed in future studies, the current body of literature suggests a key role for *rpoS* in stress response across the diverse genus *Pseudomonas*.

In repeated growth-chamber and field trials, we found that an *rpoS* mutant of Pf-5 did not survive as well as the wild-type strain in the rhizosphere of beans planted in dry soil, whereas differences in survival of the strains were sometimes less pronounced in moist soils. Therefore, the role of *rpoS* in rhizosphere fitness of Pf-5 could be attributed in part to the central role of *rpoS* in stress response, in this case the survival of desiccation stress. Similar findings have been reported for other *Pseudomonas* spp., where the role of *rpoS* in fitness has been tested in microcosms. *rpoS* influenced root colonization by a strain of *P. putida* under competitive conditions from other microbes, whereas *rpoS* did not have a detectable influence on colonization of roots planted in a sterile soil and maintained under gnotobiotic conditions (Miller *et al.*, 2001a). An *rpoS* mutant of *P. putida* KT2440 was more sensitive than the wild-type strain to stress imposed by the toxic compound *m*-methylbenzoate in soil (Ramos-González & Molin, 1998). The population size of the *rpoS* mutant declined more than that of the wild-type strain in soil treated with this compound. In contrast, soil populations of KT2440 derivatives containing the TOL plasmid, which confers *m*-methylbenzoate degradation, were stable in the soil irrespective of *rpoS*. From these results, we suggest that the role of *rpoS* as a fitness factor of *Pseudomonas* spp. in the soil or rhizosphere is related in part to its role in surviving stresses that the bacteria encounter in these habitats.

The findings of this study contribute to an evolving view of *rpoS* as an essential component of stress response in *Pseudomonas* spp., a role commonly attributed to its stature as a central regulator influencing stationary-phase gene expression. *rpoS* is known to influence the expression of at least 100 genes in *E. coli* upon conditions of cellular starvation or stress (Ishihama, 2000). Similarly, in *P. putida* KT2440, *rpoS* controls the expression of more than 50 genes encoding peptides that are synthesized in cells after a short period of carbon starvation (Ramos-González & Molin, 1998). The span of its control must certainly be one factor in the cross-protection that *rpoS* exerts in the stress response of stationary-phase cells. It must also be remembered, however, that certain protective mechanisms can enhance a cell's capacity to survive a number of individual stresses. For example, freeze-induced cellular injury is both specific (damage due to intracellular ice crystal formation) and shared with other types of stresses, such as desiccation, osmotic and oxidative stresses (Tanghe *et al.*, 2003). Because of the destructive effects of oxygen during desiccation and freezing, oxygen-scavenging mechanisms of the cell are likely to be critical to the survival of cells encountering dry environments or subzero temperatures (Potts, 1994; Tanghe *et al.*, 2003). With oxidative damage heralded as the 'Achilles heel' of stationary-phase bacterial cells (Nyström, 2004), oxygen management is likely to play a central role in a cell's capacity to survive in the presence of diverse environmental stresses, such as those imposed by starvation, freezing and desiccation. In that regard, *rpoS* is known to play a critical role in the oxidative stress response of *Pseudomonas* spp. (Jørgensen *et al.*, 1999; Miller *et al.*, 2001a; Sarniguet *et al.*, 1995; Suh *et al.*, 1999). Future studies are needed to identify mechanisms involved in the cellular response to the diverse environmental stresses that bacteria encounter in natural environments, and the role of *rpoS* and other global regulators in the capacity of the bacteria to survive these stresses.

ACKNOWLEDGEMENTS

This research was supported in part by the USDA-CSREES, National Research Initiative-Competitive Grants Program 95-37312-1655. We thank Walter Mahaffee for his thoughtful review of the manuscript.

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